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Journal of Cellular Biochemistry 113:977–984 (2012)

Journal of Cellular Biochemistry

Determinants of the Specificity of Protease-Activated Receptors 1 and 2 Signaling by Factor Xa and Thrombin

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ABSTRACT

Factor Xa (FXa) elicits intracellular signaling responses through the activation of protease-activated receptor 2 (PAR2) and possibly also through PAR1 in endothelial cells. In this study, we investigated FXa signaling in endothelial cells when the protease was either in free form or assembled into the prothrombinase complex. Furthermore, we prepared several wild-type and mutant PAR1 and PAR2 cleavage-reporter constructs in which their exodomains were fused to cDNA encoding for a soluble alkaline phosphatase (ALP). In the mutants, P2 residues were exchanged between PAR1 and PAR2 cleavage-reporter constructs and the hirudin-like binding site (HLBS) of PAR1 was inserted into the homologous site of PAR2. In non-transfected cells, FXa elicited a protective response which could be blocked by a specific anti-PAR2 but not by an anti-PAR1 antibody. A similar protective activity was observed for FXa in the prothrombinase complex. Further studies revealed that neither the Gla- nor EGF1-domain of FXa is required for its signaling activity, however, the N-terminus Arg-86 and Lys-87 of the EGF2-domain were essential. In the cleavage-reporter transfected cells, FXa cleaved the PAR2 construct effectively, however, replacing its P2-Gly with P2-Pro of PAR1 impaired its cleavage by FXa but improved it by thrombin. A PAR2 construct containing both P2-Pro and HLBS of PAR1 was poorly cleaved by FXa, but effectively by thrombin. A PAR1 construct containing P2 and P3 residues of PAR2 was poorly cleaved by thrombin but effectively by FXa. These results provide new insight into mechanisms through which coagulation proteases specifically interact with their target PAR receptors. J. Cell. Biochem. 113: 977–984, 2012.

KEY WORDS: PAR1; PAR2; THROMBIN; FXa; SPECIFICITY

Factor Xa (FXa) is a vitamin K-dependent trypsin-like serine protease in plasma which upon interaction with factor Va (FVa) on negatively charged membrane surfaces in the presence of calcium (prothrombinase complex) activates prothrombin to thrombin during the blood coagulation process [Jackson and Nemerson, 1980; Mann et al., 1988; Davie et al., 1991]. Thrombin cleaves fibrinogen to fibrin to form blood clots at the site of vascular injury, thereby preventing blood loss from injured vessels [Jackson and Nemerson, 1980; Mann et al., 1988; Davie et al., 1991]. In addition to their essential roles in the clotting cascade, both FXa and thrombin also elicit intracellular signaling responses through the activation of protease-activated receptors (PARs), expressed at the surface of endothelial cells and other cell types [Camerer et al., 2000; Riewald and Ruf, 2001; Ruf et al., 2003; Coughlin, 2005; Feistritzer et al., 2005; Mosnier et al., 2007]. PARs belong to a sub-family of G-protein coupled receptors with four members having been identified and characterized so far (PAR1, PAR2, PAR3, and PAR4) [Coughlin, 2005]. Thrombin can activate PAR1, PAR3, and PAR4, but not PAR2 [Camerer et al., 2000; Coughlin, 2005]. It appears that PAR2 is specifically cleaved by FXa and factor VIIa-tissue factor complex but not by other coagulation proteases [Camerer et al., 2000; Riewald and Ruf, 2001; Rao and Pendurthi, 2005]. Recent results have indicated that FXa can also signal through PAR1 in endothelial cells [Feistritzer et al., 2005; Bhattacharjee et al., 2008]. All cell signaling studies with FXa have been conducted with the free form of FXa. Whether the physiologically relevant form of FXa in the prothrombinase complex can also cleave PAR2 (or PAR1) to initiate signaling responses in endothelial cells has not been investigated.

The molecular basis of the specificity of cell surface PAR recognition by coagulation proteases is not fully understood. Structural data suggests that the interaction of an Asp, located at the primary specificity pocket of trypsin-like coagulation proteases, including FXa and thrombin [residue 189 in chymotrypsin numbering; Bode et al., 1989], with an Arg at the P1 site [nomenclature of Schechter and Berger, 1967] of peptide substrates plays an essential role in determining the substrate specificity of

Grant sponsor: National Heart, Lung, and Blood Institute of the National Institutes of Health; Grant numbers: HL 101917, HL 68571.

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Received 28 July 2011; Accepted 18 October 2011 • DOI 10.1002/jcb.23427 • © 2011 Wiley Periodicals, Inc.
Published online 27 October 2011 in Wiley Online Library (wileyonlinelibrary.com).

these proteases [Bode et al., 1989; Padmanabhan et al., 1993]. Since the extracellular domains of all PARs contain an Arg at P1 positions, their specificity of interaction with coagulation proteases must therefore be primarily determined by the P1-Arg of the receptor exodomains interacting with Asp-189 of these proteases through a typical bifurcated salt-bridge interaction [Bode et al., 1989; Padmanabhan et al., 1993]. In addition to P1-Arg, coagulation proteases also require specific interactions with other residues surrounding the scissile bond, or exosites remote from it, in order to engage their substrates in the catalytic reactions, a feature that is not shared by trypsin [Rezaie, 1996; Rezaie and Esmon, 1996; Krem et al., 2000; Krishnaswamy, 2005]. Thus, it has been demonstrated that a Gly at the P2 positions of substrates and inhibitors is the most preferred residue for fitting into the narrow P2-binding pocket of FXa [Djie et al., 1996; Rezaie, 1996; Chuang et al., 1999]. Among the four members of the PAR sub-family, PAR2 is the only member which has a Gly at this position, possibly accounting for its high specificity of interaction with FXa, but not with thrombin. On the other hand, a Pro at the P2 position of PAR1 is a preferred residue for the hydrophobic P2-binding pocket of thrombin [Bode et al., 1989], thus accounting for its specificity of interaction with thrombin but not with FXa. In the case of PAR1, however, the interaction of basic residues of exosite-1 of thrombin with an acidic hirudin-like binding site (HLBS) of the PAR1 exodomain, located at the C-terminus of the scissile bond, also contributes to the protease recognition of the receptor [Liu et al., 1991; Lane et al., 2005]. A binding exosite for FXa on PAR2 has not been identified and it is not known if such an exosite-dependent interaction contributes to the specificity of PAR2 recognition by FXa.

In this study, we investigated the question of the specificity and the mechanism of PAR signaling by coagulation proteases, by monitoring the signaling function of FXa, by itself or in complex with FVa in the prothrombinase complex, in endothelial cells stimulated with either thrombin or bacterial lipopolysaccharide (LPS). We also prepared several PAR1 and PAR2 cleavage reporter constructs in which the extracellular domains of the receptors were fused to a cDNA encoding for the soluble ALP. In these constructs, we exchanged the P2/P3 residues between PAR1 and PAR2 and also inserted HLBS of PAR1 to the homologous site of PAR2. The cleavage specificity of receptors by FXa and thrombin in endothelial cells, transfected with these constructs, were monitored. The cell signaling data with non-transfected cells indicated that FXa, either in free form or in complex with FVa in the prothrombinase complex, can elicit a barrier protective activity that is blocked by a specific function-blocking antibody to PAR2, but not to PAR1. The signaling function of FXa was found to be independent of its Gla-domain. The results with transfected cells indicated that the PAR cleavage specificity of FXa and thrombin can be switched if the P3/P2 residues and/or the HLBS sequence between PAR1 and PAR2 constructs have been swapped.

MATERIALS AND METHODS

MATERIALS

Human plasma proteins including FXa, FVa, thrombin, anti-thrombin, and factor X-activating enzyme from Russell's viper venom (RVV-X) were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). The chromogenic substrates, Spectrozyme FXa (SpFXa) was purchased from American Diagnostica (Greenwich, CT) and the thrombin chromogenic substrate, S2238, was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PCPS) were prepared as described [Chen et al., 2004]. LPS was purchased from Sigma (St. Louis, MO). The function-blocking anti-PAR1 (H-111) and anti-PAR2 (SAM-11) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody concentrations used in the blocking assays were 25 μg/ml in all experiments. Transformed human umbilical vein endothelial cells (HUVECs), EA.hy926 cells, were obtained from Dr. C. Edgell (University of North Carolina at Chapel Hill, NC).

CONSTRUCTION, EXPRESSION, AND PURIFICATION OF RECOMBINANT PROTEINS

Construction and expression of wild-type factor X (FX) and its mutants lacking either the Gla domain (GD-FX) or both the Gla and EGF1 domains (E2-FX) in human embryonic kidney (HEK-293) cells have been described [Manithody et al., 2002; Rezaie et al., 2004]. An E2-FX mutant containing Ala substitutions for residues Arg-86 and Lys-87 (E2-FX/RK-AA) was expressed using the same vector system. All recombinant FX derivatives were converted to active forms by established methods using RVV-X and purified to homogeneity as described [Manithody et al., 2002]. The construction and characterization of the Arg-165 to Ala (R165A) and Lys-169 to Ala (K169A) substitution mutants of FXa has been described [Rezaie, 2000]. Active-site concentrations were determined by an amidolytic activity assay using SpFXa and titrations with human anti-thrombin assuming a 1:1 stoichiometry as described [Manithody et al., 2002; Chen et al., 2004]. These concentrations were within 90–100% of those expected based on zymogen concentrations as determined from the absorbance at 280 nm using a published absorption coefficient [Manithody et al., 2002; Chen et al., 2004]. The PAR1 and PAR2 cleavage reporter plasmids were constructed by fusing the cDNA encoding for the secreted human tissue non-specific ALP [Weiss et al., 1986], lacking the last 19 COOH-terminal residues, to the N-termini of the cDNAs encoding for either PAR1 (PAR1-ALP) or PAR2 (PAR2-ALP) in the mammalian expression vector pRc/RSV (Invitrogen, San Diego, CA) as described [Bae et al., 2008]. The same vector was used to prepare a PAR1-ALP construct in which the P3 and P2 residues of PAR1 (Asp-39 and Pro-40, respectively) were replaced with the corresponding P3 and P2 residues of PAR2 (Lys-34 and Gly-35, respectively) (PAR1-ALP/D39K-P40G). The same methods were used to prepare three PAR2-ALP constructs in which (i) the P2-Gly of PAR2 was replaced with the P2-Pro of PAR1, (ii) the eight residues of the HLBS (Asp–Lys–Tyr–Glu–Pro–Phe–Trp–Glu), located at the C-terminus of the PAR1 scissile bond, were inserted into the homologous site of PAR2, and (iii) a double mutant of PAR2 which contained both the P2-Pro and the HLBS sequence of PAR1. Additionally, a PAR1 mutant (lacking the ALP reporter) was prepared for signaling studies in which the P3 (Asp-39) and P2 (Pro-40) residues of the receptor were replaced with the residues found at

Fig. 1. Partial sequence alignment of exodomains of PAR1 and PAR2. The residues swapped between the two receptors are underlined. The P1-Arg residue cleaved by coagulation proteases is shown by an arrow. The hirudin-like binding site (HLBS) of PAR1, which has not been conserved in PAR2 is shown between two vertical lines.

the identical sites of PAR2 (PAR1/D39K-P40G) (see Fig. 1 for the list of PAR1 and PAR2 fusion constructs).

PAR CLEAVAGE ASSAYS

Transformed HUVECs (EA.hy926 cells), at 90% confluence in 24-well plates, were transiently transfected with pRc/RSV containing PAR1-ALP and PAR2-ALP reporter constructs (1 μg/well) in antibiotic-free Opti-MEM medium using Lipofectamin (Invitrogen, Carlsbad, CA) as described [Bae et al., 2008]. Following 48-h incubation at 37°C in 5% CO_2, cells were washed and incubated in serum-free medium for 5 h followed by their incubation for an hour with varying concentrations of thrombin or FXa derivatives (0–100 nM). Conditioned media were collected and centrifuged to remove cell debris. Supernatant was collected and the ALP activity was measured using SensoLyte luminescent secreted ALP reporter gene assay kit (AnaSpec, San Jose, CA) according to manufacture's instruction and as described [Bae et al., 2008]. Results were expressed as mean ± SE and all experiments were repeated at least three times.

SIGNALING ASSAYS

The signaling activity of FXa derivatives was assessed by a permeability assay as described [Bae et al., 2007]. Briefly, FXa derivatives (1–100 nM), or FXa (25 nM) in complex with human FVa (50 nM), and PC/PS vesicles (25 μM) in TBS containing 5 mM $CaCl_2$ were added to confluent monolayers of EA.hy926 cells cultured in a modified two-compartment chamber model system in 12-well tissue culture plates as described [Bae et al., 2007, 2010]. The proteases were added to cells in serum-free medium for 3 h at 37°C in 5% CO_2. Cell monolayers were then washed and stimulated by either thrombin (10 nM for 10 min) or by LPS (10 μg/ml for 5 h) to induce hyper-permeability as described [Bae et al., 2007, 2010]. The barrier permeability was quantitated by the spectrophotometric measurement of the flux of Evans blue-bound albumin across the cell monolayers as described [Bae et al., 2007]. For the PARs function-blocking antibody treatments of the monolayers, medium was removed and antibodies were added for 30 min in serum-free medium followed by the analysis of the cell permeability as described [Bae et al., 2007]. Results were expressed as mean ± SE and all experiments were repeated three times.

The same procedures were employed to monitor the activity of FXa on the barrier permeability of endothelial cells in response to LPS or thrombin after transfecting cells with the expression vectors containing the cDNA for PAR1/D39K-P40G. In this case, cells were first transfected in 100 mm × 20 mm tissue culture dishes with 12–15 μg DNA using Lipofectamin as described above. Following 24 h incubation at 37°C in 5% CO_2, cells were carefully lifted from the tissue culture dishes using enzyme-free cell dissociation buffer (Invitrogen, Carlsbad) and were seeded at a high density of 4×10^5/well in 12-well trans-well plates as described above. Cells were allowed to attach to membrane for 24 h and cell permeability in response to either thrombin or LPS was conducted as described above. The permeability assay was carried out in triplicate wells.

RESULTS AND DISCUSSION

FXa SIGNALS THROUGH PAR2 IN ENDOTHELIAL CELLS

FXa is known to elicit intracellular signaling activities in response to inflammatory mediators through the proteolytic activation of the cell surface receptor, PAR2 [Riewald and Ruf, 2001; Ruf et al., 2003]. In a recent study, a potent barrier protective activity for FXa in HUVECs was observed, which appeared to be mediated through both PAR1 and PAR2 [Feistritzer et al., 2005]. We investigated this question further, and thus monitored the concentration-dependence of the barrier protective activity of FXa in EA.hy926 cells in response to thrombin. EA.hy926 cells express both PAR1 and PAR2 on their membrane surfaces. As presented in Figure 2A, FXa elicited a potent barrier protective effect in these cells by a concentration-dependent manner which peaked at ~20 nM FXa. To determine whether the protective activity of FXa is mediated through PAR1 or PAR2, the permeability assay was carried out with 25 nM FXa following incubation of cells with function-blocking antibodies to

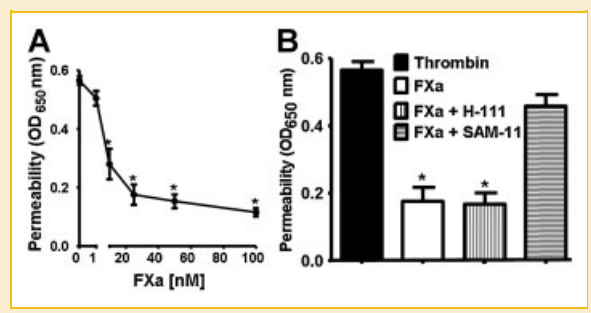

Fig. 2. Concentration-dependence and PAR2-dependence of barrier protective activity of FXa in endothelial cells. A: Confluent EA.hy926 cells in a dual chamber system were pre-incubated for 3 h with increasing concentrations of FXa, followed by inducing the permeability with 5 nM thrombin for 15 min. Permeability was assessed by the amount of BSA-bound Evans blue dye diffused into the lower chamber by measuring OD_{650} nm. Each point represents mean ± SD for three independent wells. Unpaired *t*-test suggests statistical significance with $^*P < 0.05$ versus no FXa. B: The same as (A) except that cells were pre-incubated with 25 μg/ml function-blocking anti-PAR1 (H-111), and anti-PAR2 antibodies (SAM-11) prior to treatment with 25 nM FXa for 3 h, followed by incubation with 5 nM thrombin for 15 min. Permeability was assessed as described above. Each bar represents mean ± SD for three independent wells. Unpaired *t*-test suggests statistical significance with $^*P < 0.05$ versus thrombin.

either PAR1 or PAR2. The results presented in Figure 2B indicated that the anti-PAR2 antibody abrogated all barrier protective activity of FXa, however, the anti-PAR1 antibody had no effect on this function of the protease. The anti-PAR1 antibody effectively blocked a similar PAR1-dependent barrier protective activity observed with activated protein C (APC) which was used as a positive control (data not shown), suggesting that the anti-PAR1 antibody is functionally active. This is in agreement with previous results showing that this antibody effectively blocks the PAR1-dependent signaling activities of both thrombin and APC [Bae et al., 2007, 2010]. These results suggest that signaling through PAR1 contributes minimally to the protective activity of FXa in our assay system.

To determine whether complex formation with FVa on PCPS vesicles influences the PAR2-dependent signaling activity of FXa, the same permeability assay was conducted in thrombin- or LPS-stimulated endothelial cells using 25 nM FXa, either in free form or in complex with prothrombinase (FVa, PC/PS, and Ca^{2+}). Both thrombin and LPS were used as inflammatory molecules based on the increasing evidence that coagulation and inflammation are closely linked and that thrombin and LPS can up-regulate both pathways [Ruf et al., 2003; Coughlin, 2005]. The results presented in Figure 3A indicate that both the free form of FXa and FXa in complex with FVa can elicit similar barrier protective activities in endothelial cells. Noting the high concentration of FXa required for observing a permeability effect for the protease complex, it is not known if this observation has any physiological significance. Nevertheless, the results suggest that the FVa/PCPS binding sites may not constitute interactive-sites for PAR2 binding on FXa. It should be noted that a prothrombinase assay, conducted on supernatants obtained from HUVECs, indicated that these cells do not express prothrombin to a detectable level, thus no thrombin was generated in these assays to interfere with the interpretation of results. Results of several studies have indicated that the basic residues of FXa 162-helix (Arg-165 and Lys-169) interact with FVa in the prothrombinase complex [Rezaie, 2000; Rudolph et al., 2001; Qureshi et al., 2009]. Interestingly, the Ala-substitution of these residues abrogated the activity of mutants toward PAR2-ALP, expressed on endothelial cells (data not presented). In agreement with the cleavage data, the ability of these mutants to elicit barrier protective activities in endothelial cells was also dramatically impaired (Fig. 3A). These results appear to indicate that the 162-helix of FXa interacts with PAR2. However, if the assumption that the 162-helix is an interactive-site for FVa is correct, the normal barrier protective activity of the prothrombinase complex would argue against the possibility that this helix can interact with PAR2. Thus, it is possible that the mutagenesis of the 162-helix allosterically affects other sites of FXa, thereby impairing the interaction of FXa mutants with PAR2 as observed in Figure 3A. In support of this hypothesis, a recent study has indicated that the NH2-terminal residues of the FVa-binding 162-helix are energetically linked to the S1 and Na^{+}-binding sites of FXa [Levigne et al., 2007]. Nevertheless, the Ala-substitution mutants of both Arg-165 and Lys-169 have normal amidolytic activities [Rezaie, 2000], thus further studies will be required to understand the basis for the lack of PAR2-dependent signaling activities of the 162-helix mutants of FXa.

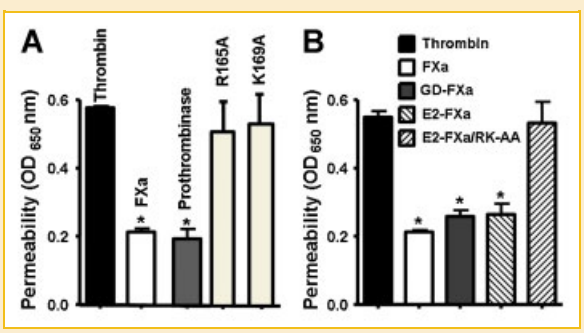

Fig. 3. Comparison of the barrier protective activity of FXa derivatives and prothrombinase in endothelial cells. A: Confluent EA.hy926 cells in a dual chamber system were pre-incubated with FXa (25 nM), prothrombinase (25 nM FXa, 50 nM FVa, and 25 μM PCPS), or the R165A and K169A mutants of FXa (25 nM) for 3 h, prior to treatment with 5 nM thrombin for 15 min. Permeability was assessed as described under the Materials and Methods Section. Each bar represents mean ± SD for three independent wells. B: Analysis of the barrier protective activity of FXa and its Gla and EGF1 deletion derivatives in the permeability assay. The same as A except that cells were pre-incubated, respectively, with 25 nM FXa, GD-FXa, E2-FXa, and E2-FXa/RK-AA for 3 h, prior to treatment with 5 nM thrombin for 15 min. Each bar represents mean ± SD for three independent wells.

ROLE OF Gla AND EGF1 DOMAINS

To investigate the role of the Gla-domain of FXa in the PAR2-dependent signaling activity of FXa, the same permeability assay was used to monitor the signaling activities of the Gla and EGF1 deletion derivatives of FXa. The results presented in Figure 3B demonstrate that both Gla-domainless GD-FXa and E2-FXa elicit a protective activity in endothelial cells with an extent similar to that observed with wild-type FXa. On the other hand, replacing the two residues Arg-86 and Lys-87 of the N-terminus E2-FXa effectively abrogated the protective signaling function of E2-FXa (Fig. 3B), suggesting that the N-terminus of EGF2-domain has an interactive-site for the receptor and/or for a cell surface co-receptor involved in mediating the PAR2-dependent signaling activity of FXa in endothelial cells. In support of a co-receptor-dependent signaling mechanism through PAR2 for FXa in endothelial cells, it has been previously reported that six residues of the inter-EGF sequence Leu-83 to Leu-88 (Leu–Phe–Thr–Arg–Lys–Leu) of FXa competes with the binding of the radio-labeled FXa to endothelial cells [Ambrosini et al., 1997]. The observation that the Ala substitution of the two basic residues, Arg-86 and Lys-87, located at the N-terminus of EGF2-domain (E2-FXa/RK-AA), led to a complete loss of signaling activity for this mutant supports the previous hypothesis that the interaction of these residues with a PAR2 co-receptor in endothelial cells is required for the signaling function of FXa [Ambrosini et al., 1997; Bae et al., 2010]. On the other hand, the observation that both free FXa and FXa in prothrombinase (the Gla-domain is occupied by PC/PS vesicles and FVa) cleaved PAR2 with similar efficiencies suggests that the interaction of the Gla-domain of FXa with an endothelial cell

surface receptor does not plays a dominant role in FXa recognition of PAR2.

CLEAVAGE SPECIFICITY OF PAR-ALP REPORTER CONSTRUCTS

To determine whether the intracellular signaling activities of FXa derivatives correlate with their ability to cleave the exodomain of PAR2 in the ALP reporter construct, the PAR1-ALP and PAR2-ALP plasmids were transiently transfected to endothelial cells and their cleavage by FXa was monitored by measuring the quantity of ALP released into the media as described under the Materials and Methods Section. Analysis of the FXa concentration-dependence of cleavage reactions indicated that FXa cleaves PAR2 significantly faster than PAR1, with the cleavage reaction saturating for PAR2 at ~70 nM protease, whereas the cleavage reaction curve for PAR1 is slower and remains linear for up to 100 nM FXa (the highest concentration of the protease tested) (Fig. 4A). The activity profiles of the FXa domain-deletion derivatives toward PAR2-ALP in the transiently transfected cells are presented in Figure 4B. Analysis of the cleavage data suggests that both GD-FXa and E2-FXa, but not E2-FXa/RK-AA can cleave PAR2, though the cleavage efficiency of the mutants was ~1.5-fold slower for GD-FXa and ~3-fold slower for E2-FXa. The cleavage of PAR2-ALP on endothelial cells by FXa was effectively blocked by the function-blocking anti-PAR2 antibody (data not shown). The basis for the slower cleavage of the PAR2-ALP construct by GD-FXa and E2-FXa is not known and was not further investigated. However, it has been reported that FXa binds to annexin 2 by a Gla-dependent mechanism to elicit intracellular signaling responses through the activation of PAR1 [Bhattacharjee et al., 2008]. Whether the higher PAR2 cleavage activity of the full-length FXa is due to its additive annexin 2-dependent cleavage of PAR2 in our over-expressing PAR2-ALP construct is not known. Moreover, it is known that the barrier permeability activity of FXa in endothelial cells is mediated through the protease cleavage of PAR2 localized to lipid-rafts [Bae et al., 2010]. Thus, it is highly possible that the PAR2 cleavage assays with the ALP over-expressing constructs are reporting the cleavage of all cell surface PAR2, however, the cell signaling assays are detecting only the cleavage of the lipid-raft localized PAR2 in endothelial cells as has been previously discussed [Bae et al., 2010].

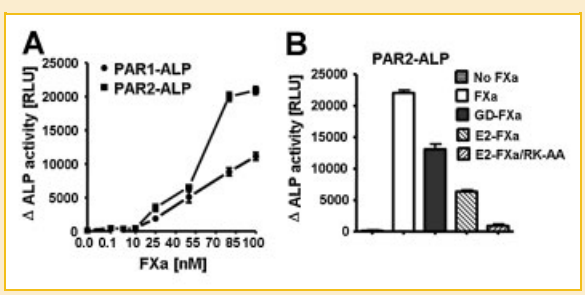

Fig. 4. Concentration-dependence of cleavage profiles of PAR1-ALP and PAR2-ALP fusion constructs by FXa. A: Confluent EA.hy926 cells, transiently transfected with either PAR1-ALP or PAR2-ALP cleavage reporter constructs, were incubated in the presence of increasing concentrations of FXa for 1 h at 37°C. The cleaved alkaline phosphatase (ALP) activity in the culture medium was measured using a chemiluminescence substrate (AnaSpec). △ALP represents the background-subtracted ALP activity in presence of the protease. Each point represents mean ± SD of ALP activity for three independent transient transfections. B: The same as (A) except that the cleavage of the PAR2-ALP reporter plasmid was monitored by FXa derivatives (100 nM each for 1 h) lacking either Gla or both Gla and EGF1 domains. Each bar represents mean ± SD of ALP activity for three independent transient transfections. RLU represents "Relative Units."

ROLES OF P2 AND P3 RESIDUES IN THE PAR RECOGNITION SPECIFICITY OF FXa AND THROMBIN

To assess the role of P2 and P3 residues in restricting the PAR specificity of coagulation proteases, we constructed a PAR1-ALP mutant in which the P3-Asp and P2-Pro of the receptor were replaced with the corresponding residues of PAR2. We monitored the time course of PAR1 cleavage by FXa on cells transfected with either wild-type PAR1-ALP or with the PAR1-ALP/D39K-P40G mutant construct. The results presented in Figure 5A suggest that, unlike the cleavage of the wild-type PAR1, FXa cleaves the mutant construct with higher efficiency. The rate of cleavage of the mutant PAR1 construct by FXa was essentially comparable to the rate of PAR2 cleavage by the protease as estimated from the comparison of the relative quantity of ALP released into the media in

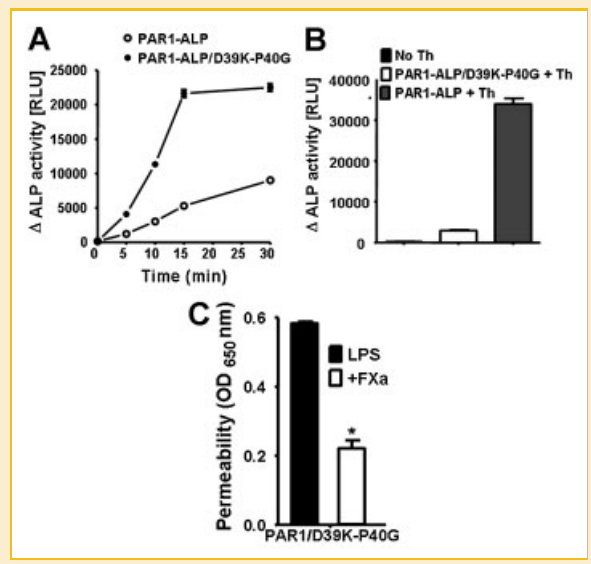

Fig. 5. Comparison of the time-dependent cleavage of wild-type and mutant PAR1-ALP constructs by FXa. Confluent EA.hy926 cells, transiently transfected with either wild-type PAR1-ALP or PAR1-ALP/D39K-P40G, were incubated with 100 nM FXa for indicated times at 37°C. The cleaved ALP activity in the cultured medium was then measured as described under the Materials and Methods Section. Each point represents mean ± SD of ALP activity for three independent transient transfections. B: Cleavage of PAR1-ALP/D39K-P40G construct by thrombin. The same as (A) except that the ALP cleavage by thrombin (10 nM for 1 h) was monitored. C: Enhanced endothelial barrier protective activity for FXa in response to LPS in cells transfected with the PAR1/D39K-P40G construct. Confluent EA.hy926 cells, transiently transfected with PAR1/D39KP40G in a dual chamber system, were pre-incubated with 5 nM FXa for 3 h, prior to treatment with 10 μg/ml LPS for 5 h. Permeability was assessed as described under the Materials and Methods Section. Each bar represents mean ± SD for three independent wells.

Figure 5A (~25,000 RLU by 100 nM FXa) and ALP released from the cleavage of wild-type PAR2-ALP construct by 100 nM FXa in Figure 4A (~25,000 RLU, 1 h cleavage). In contrast to the effective cleavage of wild-type PAR1 by thrombin, the cleavage of the PAR1-ALP/D39K-P40G mutant by thrombin was dramatically impaired (Fig. 5B). These results clearly indicate that exchanging the P3 and P2 residues of PAR1 with the corresponding residues of PAR2 effectively switches the PAR cleavage specificity of both FXa and thrombin.

To determine whether the effective PAR1 cleavage of the mutant construct by FXa correlates with its ability to elicit intracellular signaling activities, endothelial cells were transfected with an expression vector that contained PAR1 cDNA with the D39K-P40G mutation (lacking the ALP reporter) followed by monitoring the protective activity of FXa in the same permeability assay described above. As presented in Figure 5C, a significantly lower concentration of FXa (5 nM) was required to elicit a potent barrier protective effect in response to LPS in endothelial cells transfected with the PAR1 mutant. These results suggest that the cleavage of PAR1 by FXa can indeed elicit a protective response in endothelial cells, however, very high concentrations of FXa would be required to cleave threshold levels of PAR1 to be sufficient for cell signaling. It is of interest to note that the signaling specificity through either PAR1 or PAR2 is determined by the occupancy of the cell surface receptors, specific for either the Gla-domain of protein C/APC or the inter-EGF residues of FXa in the lipid-rafts of endothelial cells, as we previously demonstrated [Bae et al., 2007, 2010]. Thus, pre-treatment of endothelial cells with protein C or FX zymogens, prior to stimulation by inflammatory mediators, can alter the PAR1-dependent signaling specificity of thrombin and other coagulation proteases from a permeability-inducing and cytotoxic effect to a cytoprotective effect [Bae et al., 2007, 2010]. We have previously demonstrated that the occupancy of endothelial protein C receptor by protein C and/or the FX occupancy of an unknown receptor may alter the membrane lipid-raft localization of PAR1 and PAR2, thereby rendering intracellular signaling through both receptors protective independent of the protease cleaving the receptor [Bae et al., 2010].

ROLE OF EXOSITE-DEPENDENT INTERACTION IN DETERMINING PAR SPECIFICITY

It is known that the interaction of the basic residues of thrombin exosite-1 with the C-terminus HLBS of PAR1 makes a key contribution to the mechanism of the receptor recognition by thrombin [Liu et al., 1991]. Similar to interaction with HLBS of PAR1, the interaction of exosite-1 with other substrates and cofactors of thrombin is required for the physiological function of the protease [Liu et al., 1991; Lane et al., 2005]. To assess the extent of the contribution of HLBS to the specificity of PAR1 recognition by thrombin, the eight-residue HLBS sequence of PAR1 was inserted into the homologous site of PAR2 in the PAR2-ALP construct (PAR2-HLBS) (Fig. 1). Moreover, the P2-Gly of this PAR2 mutant construct as well as that of the wild-type PAR2-ALP was replaced with a Pro (PAR2-ALP/G35P). The cleavage profiles of thrombin and FXa with these constructs are presented in Figure 6. As expected, thrombin did not cleave PAR2-ALP with a high efficiency and the

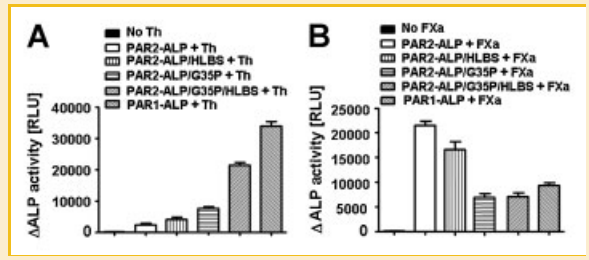

Fig. 6. The cleavage profile of thrombin toward wild-type and mutant PAR-ALP cleavage reporter constructs. A: EA.hy926 cells, transiently transfected with the indicated wild-type or chimeric PAR-ALP fusion constructs, were incubated in the presence and absence of 10 nM thrombin for 1 h at 37°C. The cleaved ALP activity in the culture medium was measured using a chemiluminescence substrate as described under the Materials and Methods Section. △ALP represents the background-subtracted ALP activity in presence of the protease. B: The same as (A) except that the cleavage profile of FXa (100 nM for 1 h) toward the cleavage reporter constructs have been monitored.

transfer of either HLSB or P2-Pro of PAR1 to PAR2 only led to a minor improvement in the efficiency of the receptor cleavage by thrombin (Fig. 6A). On the other hand, thrombin effectively cleaved the PAR2 construct which contained both HLBS and P2-Pro of PAR1, suggesting that both exosite- and active site-dependent interaction of thrombin with PAR1 is required for its optimal cleavage. The observation that the thrombin cleavage rate of the double PAR2 mutant is only ~1.5-fold slower than that of wild-type PAR1 suggests that the HLBS sequence together with the P2-site residue are the main determinants of the specificity of receptor recognition by thrombin. The results presented in Figure 6B for FXa with these mutants demonstrate that the insertion of the HLBS element of PAR1 to PAR2 minimally affects the cleavage rate of the mutant by FXa, suggesting that the exosite-dependent interaction of FXa with this site of PAR2 does not contribute to the specificity of receptor recognition by this protease. However, the substitution of the P2-Gly of PAR2 with the P2-Pro of PAR1 led to ~3-fold reduced cleavage of the PAR2 mutant by FXa. FXa cleaved the P2-Pro mutant of PAR2 and wild-type PAR1 with essentially similar lower efficiencies, suggesting that P2-Gly is the most important residue in determining the PAR specificity of FXa. By contrast, swapping the P2 and P3 residues of PAR1 with the corresponding residues of PAR2 led to a dramatic loss of the affinity of the PAR1 mutant for interaction with thrombin. However, FXa cleaved PAR1-ALP and PAR2-ALP/G35P with equal efficiency that was only ~3-fold slower than the cleavage of PAR2. Thus, relative to thrombin, FXa has a broader PAR specificity and, unlike thrombin, it neither requires an exosite for its interaction with PAR2 nor exhibits preference for the P3 residue. It should be noted that differences in the transfection efficiency between different constructs cannot account for these results since the transfections were repeated several times for all reporter constructs, and each reporter construct yielded consistent and reproducible results. Moreover, we have established similar transfection efficiency for the PAR cleavage reporter constructs in endothelial cells using our transfection protocol [Bae et al., 2008]. Thus, four different PAR1-ALP transfections yielded 50–51.6 pM ALP (soluble ALP released into the supernatant) when all cell surface

reporter construct was cleaved by excess thrombin (10 nM) for 3 h [Bae et al., 2008].

The molecular basis for the preference of FXa for a Gly at the P2 position is thought to be due to the presence of three bulky residues at the extended P2-binding pocket of FXa including Tyr-99, Phe-174, and Trp-215, which spatially restrict the specificity of this pocket in FXa to a small residue like Gly [Padmanabhan et al., 1993]. In agreement with this observation, the FXa physiological substrate (prothrombin) and inhibitor (anti-thrombin) both possess a Gly at the P2 position. In addition to the P2-specificity determining residues listed above for FXa, two residues of the B-insertion loop of thrombin, Tyr-60a and Trp-60d, protrude into the P2-binding pocket of the protease, rendering this pocket of thrombin highly specific for Pro, with the pyrrolidine ring making stabilizing hydrophobic interactions in this pocket [Bode et al., 1989]. In addition to the B-insertion loop, residue 192 of thrombin is a Glu which further restricts the specificity of the P3-binding pocket of thrombin [Rezaie and Esmon, 1996]. However, the B-insertion loop of thrombin has not been conserved in FXa, and similar to trypsin, residue 192 is a Gln in FXa [Rezaie and Esmon, 1996]. These structural features appear to contribute to the broader substrate specificity of FXa, rendering the protease capable of cleaving PAR2 independent of a secondary binding exosite, as is required for the efficient cleavage of PAR1 by thrombin. These results are in agreement with the previous observation that, unlike thrombin but similar to trypsin, FXa has less selectivity toward the P and P′ residues of the peptide substrates, with the active site groove residues of the protease tolerating a wide range of residues at these positions of the peptide substrates [Bianchini et al., 2002].

In summary, we have demonstrated in this study that the protective signaling activity of FXa is primarily mediated through its activation of PAR2 by a mechanism that is independent of the Gla-domain, but dependent on its interaction with a co-receptor through basic residues of the N-terminus EGF2-domain. Both exosite- and active site-dependent interactions dictate the specificity of PAR1 recognition by thrombin, however, only active site-dependent interaction appears to be critical for determining the specificity of PAR2 recognition by FXa and that, next to the primary specificity pocket, the P2-binding pocket is the other most significant contributor to the specificity of receptor activation by FXa. Both free FXa and FXa assembled into the prothrombinase complex are capable of initiating protective cell signaling responses in the cultured endothelial cells.

ACKNOWLEDGMENTS

We would like to thank Audrey Rezaie for proofreading the manuscript. National Heart, Lung, and Blood Institute of the National Institutes of Health, Grants HL 101917 and HL 68571 to A.R.R.

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